Role of *Phytophthora infestans* protease inhibitors and their target tomato proteases in disease

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Background and Objectives

The oomycete *Phytophthora infestans* causes late blight, a ravaging disease of potato and tomato. *P. infestans* is a hemibiotrophic pathogen that requires living host cells to establish a successful infection. Suppression of host defenses by *P. infestans* is thought to be a key pathogenicity mechanism, but remains poorly understood. We used data mining of *P. infestans* sequence databases to identify 18 extracellular protease inhibitor genes, belonging to two major structural classes: (i) Kazal-like serine protease inhibitors (EPI1 to EPI14) and (ii) cystatin-like cysteine protease inhibitors (EPIC1 to EPIC4). We hypothesize that *P. infestans* secretes these EPI and EPIC proteins to inhibit host proteases and facilitate infection. The overall objective of this project is to characterize the *P. infestans* protease inhibitors and their target tomato proteases to establish the role of these molecules in disease progression. The specific objectives are to: (i) complete the molecular characterization of serine and cysteine protease inhibitors secreted by *P. infestans*; (ii) identify and characterize the tomato proteases targeted by these inhibitors; and (iii) test specific hypotheses regarding the role of the inhibitors and their target proteases in disease.

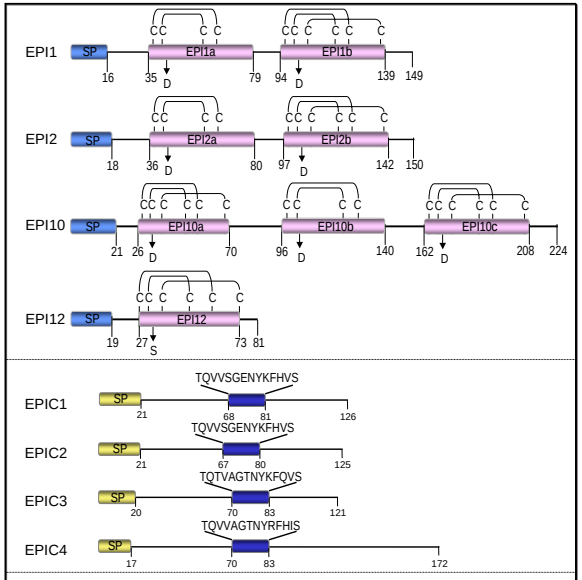

Fig. 1 Kazal domain arrangements in serine protease inhibitors EPI1, EPI2, EPI10, and EPI12 (top panel) and the conserved cystatin motif in cysteine protease inhibitors EPIC1-4 (bottom panel); SP = signal peptide

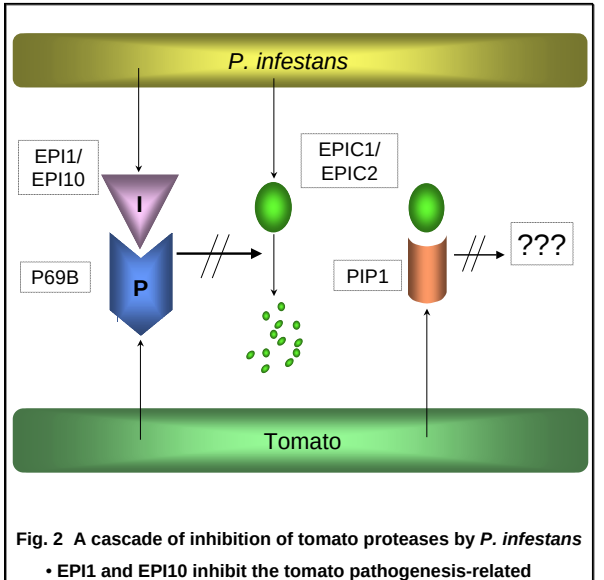

Fig. 2 A cascade of inhibition of tomato proteases by *P. infestans*

- **EPI1 and EPI10 inhibit the tomato pathogenesis-related serine protease P69B and inhibit degradation of EPIC1 and EPIC2**
- **EPIC2 interacts with tomato papain-like protease PIP1**
- **I = Protease inhibitors; P = Proteases**

Results

Eight EPIs and EPICs were expressed in *Escherichia coli* and affinity purified as fusion proteins with the epitope tag FLAG. Among the Kazal-like serine protease inhibitors, EPI1 and EPI10 interact with and inhibit the pathogenesis-related (PR) P69B subtilisin-like serine protease of tomato (Tian et al. 2004, 2005). We also extended our biochemical analyses to EPIC1-4, a new family of *P. infestans* secreted proteins with similarity to cystatin-like protease inhibitor domains. EPIC1 and EPIC2B were unstable in tomato apoplastic fluids and were degraded by tomato P69B but EPI1 protected both proteins from degradation. Coimmunoprecipitation experiments revealed that EPIC2B interacts with a novel papain-like extracellular cysteine protease, termed Phytophthora Interacting Protein 1 (PIP1). Characterization of PIP1 revealed that it is a PR protein closely related to Rcr3, a tomato apoplastic cysteine protease that functions in fungal resistance. Altogether, our findings suggest that a cascade of inhibition of host proteases initiated by EPI1 occurs in the tomato apoplast during infection by *P. infestans*. In addition, this study provides biochemical evidence for a direct contribution of the PR protein P69B subtilase to defense through the degradation of proteins from invading pathogens. We are now completing the biochemical validation of the inhibition cascade model, and have engaged in genetic analyses to determine the role of the inhibitors and their target proteases in disease.

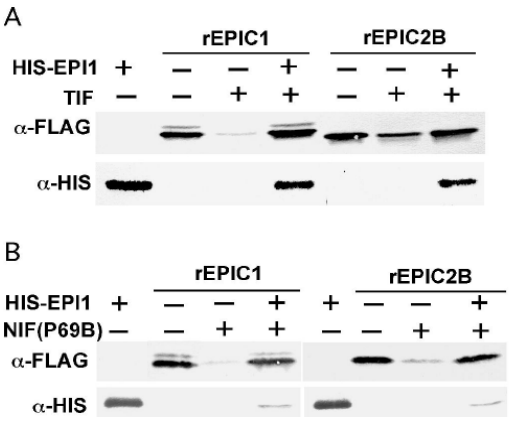

Figure 3. The Kazal-like protease inhibitor EPI1 protects EPIC1 and EPIC2B from degradation in tomato intercellular fluids

(A) EPI1 protects EPIC1 and EPIC2B from degradation in intercellular fluids from tomato. rEPIC1 and rEPIC2B were incubated with BTH-treated tomato leaf intercellular fluids (TIF +) pre-incubated with or without HIS-EPI1. HIS-EPI1, rEPIC1 and rEPIC2B without the addition of intercellular fluids were used as controls. The compositions of the reaction mixes for both rEPIC1 and rEPIC2B are indicated by the +/- signs. All the samples were electrophoresed in SDS-PAGE gel followed by immunobloting using both FLAG (a-FLAG) and HIS (a-HIS) antisera.
(B) EPI1 protects EPIC1 and EPIC2B from degradation in intercellular fluids from P69B expressing *N. benthamiana*. rEPIC1 and rEPIC2B were incubated with intercellular fluids from N. benthamiana expressing P69B (NIF +) pre-incubated with or without HIS-EPI1. HIS-EPI1, rEPIC1 and rEPIC2B without the addition of intercellular fluids were used as controls. The compositions of the reaction mixes for both rEPIC1 and rEPIC2B are indicated by the +/- signs. All the samples were electrophoresed in SDS-PAGE gel followed by immunobloting using both FLAG (a-FLAG) and HIS (a-HIS) antisera.

Future work

- Demonstrate inhibition of PIP1 by EPIC1 and EPIC2
- Biochemical characterization of P69B activity
- Genetic evidence for a role of the protease inhibitors in virulence and the proteases in defense

Acknowledgements

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Publications

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